The *Botanophila–Epichloë* association in cultivated *Festuca* in Oregon: evidence of simple fungivory

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Abstract

We investigated the *Botanophila* (Diptera: Anthomyiidae)–*Epichloë* (Ascomycetes: Clavicipitaceae) interaction in cultivated *Festuca* spp. (fine fescue) in Oregon in western USA. *Epichloë* spp. are endophytic fungi of grasses in the subfamily Pooideae. They develop a felt-like stroma on the surface of grass culms and a dense mycelium within the culms that typically prevents seed head emergence. As a result, seed yields are suppressed, and hence the disease is known as choke. Studies of *Epichloë* spp. on wild grasses indicate that the fly–fungus interaction is an obligatory mutualism. During oviposition, *Botanophila* transfers *Epichloë* spermatia between stromata of opposite mating types, and the perithecia that develop after fungal fertilization serve as food for *Botanophila* larvae. In the current study, we surveyed 19 cultivated fields of *Festuca* spp. in Oregon, and observed choke caused by *Epichloë festucae* Leuchtmann, Schardl and Siegl in 10 of these. However, perithecia were observed in only four fields, and on only 1.0–2.6% of stromata. Perithecial development was also low, and rarely covered 50% of the stroma surface. Despite the absence or low frequency of fertilized stromata, *Botanophila lobata* Collin larvae were present in all choke-infested fields. Infestation levels ranged from 2.5 to 70.7%, based on an examination of 175–200 stromata from each field. Only eight (= 2%) of the 450 stromata with *B. lobata* had perithecia, and the greater majority of *B. lobata* larvae completed development and exited from unfertilized stromata. This is the first report of the *B. lobata*–*E. festucae* association in the USA, and of *B. lobata* larvae developing successfully on unfertilized *Epichloë* stromata. The average pupal weight (0.0032 g) did not differ significantly from pupae (0.0030 g) originating from larvae that had developed on fertilized stromata of *E. typhina* on *Dactylis glomerata* in a neighboring field. This result indicates that in cultivated fine fescue fields in Oregon, *B. lobata* forages on *E. festucae*, but fly development is not dependent on the fertilized stromata of *Epichloë*.

Introduction

The association of flies in the genus *Botanophila* (previously *Anthomyia spreta* Meigen; *Phorbia phrenione* Seguy) (Diptera: Anthomyiidae) with fungi in the genus *Epichloë* (Ascomycetes: Clavicipitaceae) has been recognized for over a century (Giraud, 1872). *Epichloë* spp. are endophytic fungi of grasses in the subfamily Pooideae (White, 1987). They develop a felt-like stroma on the surface of grass culms and a dense mycelium within the culms that prevents the emergence and development of the seed head, and hence the infection by *Epichloë* is known as choke disease

(Sampson & Western, 1954). The life history and development of the fly on *Epichloë* in Europe has been described by Giraud (1872) and Lucas (1909), while Williams (1971) provided a first report of the fly–fungus interaction in the USA. Kohlmeyer & Kohlmeyer (1974) examined *Epichloë* on fresh and herbarium specimens of over 50 cool season grass species from 17 countries, and documented a widespread fly–fungus association. They reported that conidia fed upon by adult flies were viable after defecation, which suggested that the fly could transmit the fungus to new grass hosts.

In natural habitats in mid-western USA, studies by Bultman & White (1988), Bultman et al. (1995), and Bultman et al. (1998) on the fungus *E. elymi* on the native species, Canada wild rye (*Elymus canadensis* L.) and Virginia wild rye (*E. virginicus* L.), provided evidence of mutualism

between the fly and the fungus. *Epichloë* spp. are heterothallic (self-incompatible), and spermatia need to be transferred between different mating types for fertilization to occur (White & Bultman, 1987). Bultman & White (1988), Bultman et al. (1995), and Bultman et al. (1998) observed that female *Botanophila* spec. dragged their abdomen across the fungal stromata during oviposition, and defecated viable spermatia. They speculated that the fungus was fertilized during this process. Their observations indicated that perithecial development, which occurs after fertilization of the fungus, was greater in the presence than in the absence of the fly. While perithecia were consumed by *Botanophila* spec. larvae (Parker & Bultman, 1991), the benefit of spermatia transfer was considered to be greater than the loss to larval feeding, and hence the *Botanophila–Epichloë* interaction was viewed as an obligatory mutualism. *Botanophila* spec. has been reported in association with other *Epichloë* spp. (*E. amarillans*, *E. baconii*, *E. bromicola*, *E. clarkii*, *E. festucae*, *E. sylvatica*, and *E. typhina*) in natural habitats, and the fly–fungus interactions have been considered to be mutualistic (Pawlitz & Bultman, 2000; Bultman & Leuchtmann, 2003; Leuchtmann, 2003). The genus *Botanophila* is undergoing revision (GCD Griffiths, pers. comm.), and the identities of the flies associated with the various *Epichloë* fungi are not known.

A recent study by Rao & Baumann (2004) in Oregon on the west coast of USA indicated that *Epichloë* fertilization was not necessarily dependent on the presence of *Botanophila*. *Epichloë typhina*, presumably introduced from Europe, was first reported in cultivated fields of *Dactylis glomerata* (= orchard grass, cocksfoot) in western Oregon in 1996 (Alderman et al., 1997; Pfender & Alderman, 1999). The fungus spread widely, and choke is now present in nearly all orchard grass fields of the Willamette Valley in western Oregon (S. Rao, pers. obs.). Rao & Baumann (2004) observed that larvae of *Botanophila* spec. (subsequently identified as *B. lobata* Collin by D.M. Ackland, University Museum of Natural History, Oxford, UK) fed and developed on fertilized stromata of *E. typhina*. However, field observations and an exclusion study indicated that the fertilization of *E. typhina* occurred irrespective of the presence of the fly (Rao & Baumann, 2004). In one cultivated orchard grass field, all stromata were covered (>87% of each stroma) with orange perithecia (indicating that fertilization had occurred), but no fly eggs or larvae were detected in the field in two consecutive years. Hence, when *E. typhina* is present in abundance, as it is in cultivated orchard grass fields in western Oregon, it does not appear to be dependent on *Botanophila* spec. for fertilization. It continues to serve as a food source for *B. lobata* larvae, suggesting that a shift has occurred in the fly–fungus interaction, from mutualism to one of simple foraging on the fungus by fly larvae.

To further understand the *Botanophila–Epichloë* interaction in cultivated grasses, a study was conducted in fine fescue fields in western Oregon. Fine fescues are raised for seed production on over 8000 ha in Oregon (USDA-ODA, 2002), primarily in the Silverton Hills area in the Willamette Valley. The fine fescue complex includes several species of *Festuca*, including *F. rubra* ssp. *commutata* Gaudin (chewings fescue), *F. rubra* ssp. *rubra* Gaudin (creeping red fescue), *F. longifolia* Thiull (hard fescue), and *F. ovina* L. (sheep fescue), commonly used in commercial and residential turfs (Saha et al., 1987). *Festuca* spp. are reported to be infected by a single *Epichloë* species, *E. festucae* Leuchtmann, Schardl and Siegel, which is native to Europe (Leuchtmann et al., 1994). The fungus undergoes both sexual and asexual life cycles on its host (Schardl & Moon, 2003). Like *E. typhina*, *E. festucae* is sexually self-incompatible, requiring the transfer of spermatia of opposite mating types for fertilization to occur (Leuchtmann et al., 1994; Schardl, 2001). However, unlike *D. glomerata* infected with *E. typhina*, fine fescue infected with *E. festucae* can be asymptomatic and produce no stroma, and the systemic infection can extend into developing seeds and be transmitted vertically and asexually to the next generation (Schardl, 2001).

Several reports from Europe indicate the presence of stroma-producing *E. festucae* in the absence of fertilization (Sampson, 1933; Leuchtmann et al., 1994; Bazely et al., 1997; Zabalgogeazoa et al., 1998). If the fly–fungus interaction is one of obligatory mutualism and the fly larvae are dependent on perithecia for development, we would expect that *Botanophila* spec. larvae would not be associated with *E. festucae* when it is not fertilized. Alternatively, if *Botanophila* spec. larvae complete their development on unfertilized stromata, the mutualistic nature of the fly–fungus interaction would be in question. *Botanophila* spec. may contribute to fungal fertilization, but may not be dependent on its occurrence for development of the larvae. At present, while *E. festucae* is known to be associated with *Botanophila* spec. in Europe (Leuchtmann, 2003), there are no details on the nature of the association. The present study was conducted on choke in *Festuca* spp. in cultivated fields in western Oregon to determine the existence, nature, and consequences of a *Botanophila–Epichloë* interaction.

Materials and methods

Survey of seed production fields
Nineteen fields (= sites) of cultivated *Festuca* spp. in western Oregon were surveyed in June 2004 to determine the presence of *Epichloë* and *Botanophila* species (Table 1). The fields were located in the Silverton Hills area in the Willamette Valley, between 44°48.668 and 44°59.053N and 122°40.638 and 122°56.648W (GPS coordinates; Garmin

Table 1 Incidence of *Epichloë festucae* and *Botanophila lobata* in cultivated *Festuca* spp. in western Oregon

Site	Cultivated fine fescue	Incidence of *E. festucae*	No. of tillers examined	% choked tillers	No. of stromata examined	% fertilized stromata	% stromata infested with *B. lobata**[*]
1	*F. rubra* ssp. *commutata*	Present	na	na	191	2.6	9.4
2	*F. rubra* ssp. *commutata*	Absent	na	na	na	na	na
3	*F. rubra* ssp. *commutata*	Present	na	na	175	0.0	16.6
4	*F. rubra* ssp. *commutata*	Absent	na	na	na	na	na
5	*F. rubra* ssp. *commutata*	Present	4989	2.9	200	0.0	12.0
6	*F. rubra* ssp. *commutata*	Absent	na	na	na	na	na
7	*F. rubra* ssp. *commutata*	Present	6199	0.7	200	2.0	29.0
8	*F. rubra* ssp. *commutata*	Present	7481	0.5	198	1.0	70.7
9	*F. rubra* ssp. *commutata*	Present	8942	1.2	200	0.0	2.5
10	*F. rubra* ssp. *commutata*	Absent	na	na	na	na	na
11	*F. rubra* ssp. *commutata*	Present	na	na	200	1.0	8.5
12	*F. rubra* ssp. *rubra*	Absent	na	na	na	na	na
13	*F. rubra* ssp. *rubra*	Absent	na	na	na	na	na
14	*F. rubra* ssp. *rubra*	Present	na	na	200	0.0	5.5
15	*F. rubra* ssp. *rubra*	Absent	na	na	na	na	na
16	*F. rubra* ssp. *rubra*	Present	8702	1.2	200	0.0	25.5
17	*F. rubra* ssp. *rubra*	Present	na	na	194	0.0	26.8
18	*F. ovina*	Absent	na	na	na	na	na
19	*F. longifolia*	Absent	na	na	na	na	na

[*]Stromata with *B. lobata* eggs, larvae or empty larvae cases.

GPS III Plus, Garmin International, KS). Fields of *F. rubra* ssp. *commutata*, *F. rubra* ssp. *rubra*, *F. longifolia*, and *F. ovina* were examined. All fields had been in seed production for 1–6 years, and ranged in size from 2 to 36 ha. The survey was conducted within 1–2 weeks of harvest.

Incidence of choke

Tillers were collected at 10 random locations, 9 m apart, along each of two transects, each 90 m long, from sites 5, 7, 8, 9, and 16. All tillers within a grid 1×0.25 m were collected. The total number of healthy tillers and the number of tillers with choke were recorded for each site.

Fly–fungus interaction

Fifty tillers with symptoms of choke were collected at random along each of four transects, 90 m long, arranged in a diamond pattern in each choke-infected field (Table 1). Choked tillers (175–200) collected from each field were examined under a dissecting microscope. Records were made of: (1) the number of stromata fertilized; (2) the number of choked tillers infested with *Botanophila* spec.; (3) number of *Botanophila* spec. eggs, larvae, or larval cases on each stroma. Observations were also made on the food resource (fungal or plant material) used by the fly larvae. Larvae that exited the stromata were maintained in Petri dishes with moist sand for pupation. Voucher specimens were deposited in the Oregon State Arthropod Collection, Oregon State University, Corvallis, Oregon, USA.

Botanophila spec. larvae were collected from stromata, preserved in 95% ethanol, and sent to A. Leuchtmann (Geobotanisches Institut ETH, Zurich, Switzerland) for inclusion in a DNA sequence analysis of fly larvae collected from *Epichloë* in Switzerland. For comparison, *Botanophila* spec. larvae from *E. typhina* stromata from a seed production field of *D. glomerata* 3 km from an infested fine fescue field were also sent for analysis. A random sample of 50 *Botanophila* spec. pupae from each of the two fungal hosts were weighed and compared using a t-test.

In a separate study, data were collected to determine if there was a relationship between stroma length and the number of *Botanophila* spec./stroma. Four fields sown with chewings fescue (Table 1: sites 5, 7, 8, and 9) were sampled. In each field, all choke-infested tillers were collected from a 1 m² area from a minimum of eight random locations that were at least 150 m apart. At site 8, however, choked tillers were located at one end, thus sampling was confined to this area. Linear regression analysis was used to determine if there was a correlation between stroma length and number of *Botanophila* spec. present.

Results

Survey of seed production fields

The fungus was identified as *E. festucae* by C. Schardl (University of Kentucky, USA). It was present in 10 of 19 (= 52.6%) cultivated fields surveyed in 2004. Seven of

these fields were of *F. rubra* ssp. *commutata*, and three were of *F. rubra* ssp. *rubra* (Table 1). No choke was found in any of the *F. ovina* or *F. longifolia* fields included in the survey.

Incidence of choke

At the five sites from which healthy and choked tillers were collected for estimation of the incidence of choke, we collected a total of 4989–8942 tillers per field. *Epichloë festucae* stromata were observed on 0.7–2.9% of the tillers (Table 1).

Fly–fungus interaction

Based on DNA sequence analysis, A. Leuchtmann identified the larvae collected from *E. festucae* as *B. lobata* (A. Leuchtmann, pers. comm.). *Botanophila lobata* was present in all 10 fields harboring *E. festucae* stromata, but was highly variable in its abundance (Table 1). Overall, it was present on 405 (= 20.7%) of the 1958 stromata examined. The percentage of stromata per field that had *B. lobata* eggs, larvae, or larval cases ranged from 2.5 to 70.7% (Table 1). Stromata had 0 (79.3%), 1 (17.1%), 2 (2.6%), or >2 (1.0%) eggs or larvae (Figure 1).

Eggs were <1 mm long, white, elongate, sculptured, and attached to the stromata. Larval cases were typically up to 1–1.5 cm long, constructed partly from fecal material, either with or without a larva inside. Larvae were observed feeding on unfertilized stromata and grass tissue below the stromata. Pupation occurred after the larvae dropped from a stroma.

Perithecial development was observed only late in the season, and at only four sites, all chewings fescue fields (Table 1). Fertilization levels were low, ranging from 1 to 2.6% of stromata in a field, and covering <50% of the stroma surface. Only eight (= 2%) of the 405 stromata that had *B. lobata* feeding on them had evidence of perithecial development.
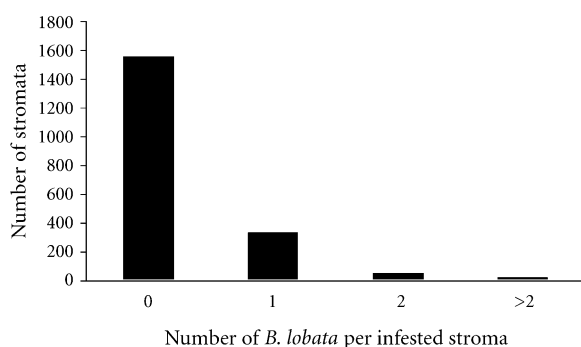
Figure 1 Distribution of *Botanophila lobata* (eggs, larvae, and empty larval cases) on *Epichloë festucae* stromata from 10 cultivated *Festuca rubra* fields in western Oregon. Total number of stromata examined = 1958.
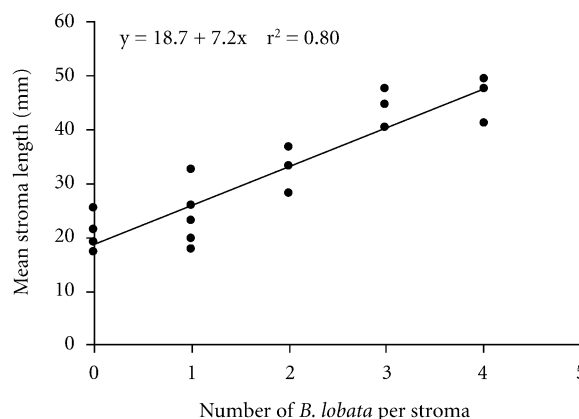
Figure 2 Correlation between the mean stroma length per field and number of *Botanophila lobata* on the stromata in four cultivated *Festuca rubra* ssp. *commutata* fields in western Oregon. Total number of stromata examined = 1754.

All larvae collected from the stromata of *E. typhina* on *D. glomerata* were observed feeding on perithecia. DNA sequence analysis indicated that the larvae belonged to *B. lobata* (A. Leuchtmann, pers. comm.). There was no significant difference in weight of the pupae that developed on unfertilized stromata of *E. festucae* (mean $\pm$ SE = 0.0032 $\pm$ 0.0001 g) and on fertilized stromata of *E. typhina* (0.0030 $\pm$ 0.0001 g) (t-test: t = 1.27, P = 0.21).

The lengths of 1754 stromata from four *Festuca* fields ranged from 5 to 67 mm, with 20.04 mm $\pm$ 0.19 mm as the average. There was a positive correlation between mean stroma length per field and the number of *B. lobata* present ($F_{1,12}$ = 47.27, P<0.0001) (Figure 2). Across all four fields, mean stroma length was 20.25 mm $\pm$ 1.99 mm when there was no *B. lobata*, 23.20 mm $\pm$ 3.29 mm when there was one, 32.29 mm $\pm$ 4.17 mm when there were two, and 45.57 mm $\pm$ 1.73 mm when >2 were present. The longest stromata were observed at site 8, which also had the highest fly density, and greater numbers of stromata with two or more *B. lobata*.

Discussion

The current study is the first to report *E. festucae* infesting *Festuca* spp. in Oregon. Fine fescues have been raised commercially for seed in the Willamette Valley in western Oregon since the early 1930s (G. Gingrich, pers. comm.). The first record of choke in chewings fescue in the Willamette Valley is April 1990 (Plant Clinic, Oregon State University). It may have been introduced earlier but remained unreported as it is not very visible, and choke is not a great economic concern in fine fescue seed production. We estimated the incidence of choke in five fine fescue

fields and recorded a maximum of 2.9% choked tillers. In contrast, *E. typhina* on *D. glomerata* is more striking due to its larger size and the presence in abundance of bright orange perithecia. Pfender & Alderman (1999) reported that up to 28% of *D. glomerata* tillers were affected by choke in the Willamette Valley. Sampson (1933) also reported that *E. typhina* was more pathogenic than *E. festucae* in Europe in terms of its impact on seed production.

Epichloë festucae is known to infest *Festuca* spp., *Lolium* spp., and *Koeleria cristata* (Leuchtmann et al., 1994; Moon et al., 2000; Craven et al., 2001). According to Zabalgogeazoa et al. (1998), commercial cultivars of *F. rubra* are rarely endophyte-infected. In contrast, in our study we observed *E. festucae* stromata in 58.8% of commercial *F. rubra* fields surveyed. However, while four *Festuca* species were examined, fungal stromata were detected only on *F. rubra* ssp. *commutata* and *F. rubra* ssp. *rubra* (Table 1). *Epichloë festucae* may have been present asymptomatically in *F. ovina* and *F. longifolia*, but this was not investigated as our focus was on the fly–fungus interaction. Nonstroma-forming *Epichloë* have been reported on *F. longifolia* and *F. ovina* (Saha et al., 1987; Leuchtmann, 1992), and further studies will be needed to determine its presence in these two *Festuca* spp. in Oregon.

Epichloë festucae stromata were present in 10 fine fescue fields (seven chewings fescue and three creeping red fescue), but perithecia were observed in only four fields, all chewings fescue, late in the season. This contrasts with the *E. typhina–D. glomerata* association in Oregon where fertilization was present on all stromata examined (Rao & Baumann, 2004). The absence or low levels of fertilization in *E. festucae* have already been reported (Sampson, 1933; Leuchtmann et al., 1994; Bazely et al., 1997; Zabalgogeazoa et al., 1998). Zabalgogeazoa et al. (1998) suggested that low fertilization in *E. festucae* may be the result of an asymmetric distribution of mating types. Further research is needed to determine whether *E. festucae* in Oregon is dominated by one mating type. Irrespective of the basis for the low fertilization, development of *B. lobata* larvae on unfertilized stromata is significant, as it indicates that the fly–fungus interaction is not one of obligatory mutualism in cultivated *F. rubra* fields in Oregon. Fungal fertilization during oviposition may be serendipitous but not obligatory for the development of *B. lobata* larvae.

This is the first report on the interaction of *B. lobata* with *E. festucae* in the USA. Kohlmeyer & Kohlmeyer (1974) reported the presence of a single *Botanophila* spec. on a fungal stroma on a herbarium specimen of *F. nutans* Spreng. (= *F. paradoxa* Desv., see Soreng et al., 2003) from Indiana. *Botanophila* spec. larvae have been observed in association with *E. festucae* in Kentucky, but the study is not yet published and no observations were made on the interaction

(C. Schardl, pers. comm.). Earlier, Schardl & Leuchtmann (1999) reported that flies ignored stromata of *E. festucae* although they visited *E. elymi* on the same farm in Kentucky. In Europe, of the five *Botanophila* spp. associated with *Epichloë* (Ackland, 1972), more than one is associated with *E. festucae* (Leuchtmann, 2003). However, their specific identities are not known, and no studies have examined their role in *E. festucae* fertilization.

In Europe, *Botanophila* spp. have been observed to be *Epichloë* species-specific in field studies, although not in cage studies (Bultman & Leuchtmann, 2003). We observed *B. lobata* feeding on two fungal hosts, *E. festucae* and *E. typhina* in Oregon, though the fly–fungus interaction differed on the two hosts. In *E. festucae*, of the 405 stromata that were infested with *B. lobata*, only 17.3% had two or more eggs or larvae per stroma and the maximum number on a stroma was four. In contrast, over 50% of infested stromata were observed to have two or more fly larvae on *E. typhina* (Rao & Baumann, 2004). The difference can be correlated with stroma length in the two fungi. The longest stroma in *E. festucae* was 6.7 cm, whereas *E. typhina* stromata reached 15 cm and harbored up to 10 fly larvae. These results coincide with the study by Pawlitz & Bultman (2000), in which the frequency of two or more eggs per stroma was lower on *E. baconii*, with a mean stroma length of 0.96 cm, than on *E. clarkia*, with a mean stroma length of 2.21 cm.

The greatest difference between fly–fungus interactions involving *E. typhina* and *E. festucae* in Oregon relates to the fungal resource used by fly larvae for development. Even with no or low level fertilization, *B. lobata* was associated with *E. festucae* in all 10 choke-infested fine fescue fields included in the survey, and fly larvae developed on and exited from unfertilized stromata. Clearly, the larval development of *B. lobata* was not dependent on *E. festucae* fertilization in cultivated fine fescue fields in Oregon. In contrast, fly larvae developed and exited from the fertilized stromata of *E. typhina* (Rao & Baumann, 2004). However, although *E. typhina* fertilization was widespread, *B. lobata* was not present in all choke-infested fields. Bultman & Leuchtmann (2003) and Leuchtmann (2003) speculated that *Botanophila* spec. larvae depended on fertilized stromata as a food source. Larvae have been observed developing successfully on stromata with little perithecia (T. Bultman, pers. comm.), but this has not been reported. The current study is the first report of *B. lobata* larvae completing development on unfertilized stromata. We observed no difference in pupal weight between *B. lobata* larvae that developed on the fertilized stromata of *E. typhina* and unfertilized stromata of *E. festucae*. Further research is needed to determine if other aspects of development, such as survivorship, differ depending on whether the larvae feed on perithecia or unfertilized stroma.

This research corroborates the earlier finding that the interaction of *B. lobata* with E. *typhina* in cultivated orchard grass seed production fields in Oregon is one of simple foraging by the fly on the fungus (Rao & Baumann, 2004). Earlier studies that have provided evidence of mutualism in the fly–fungus interaction were conducted in natural habitats. In such environments, *Epichloë* spp. are patchy in distribution (Williams, 1971; Leuchtmann, 1992), and the role of the fly may be critical for fungal fertilization. However, when the fungus is present in abundance in cultivated grass seed fields, *Epichloë* appears to be exploited by endemic *B. lobata*.

The *Botanophila–Epichloë* interactions in cultivated grasses in Oregon raise interesting evolutionary questions. At present, *B. lobata* is reported from Europe and Japan (Collins, 1967; Suwa, 1999), and we are awaiting the revision of the genus *Botanophila* in North America by G.C.D. Griffiths for insights on the origin of *B. lobata* in Oregon. If the fly was introduced to Oregon from Europe, either as eggs or larvae on the stromata of one of the exotic *Epichloë*, or as pupae in soil, decoupling of *B. lobata* egg laying and fungal fertilization could have occurred in the new environment, either due to a lag in time between introduction of the fungus and the fly or the commercial field environment with high densities of grass and fungus. If, however, *B. lobata* is a holarctic species, and is native to Oregon, the local population would appear to have switched from a native fungus to the exotic *E. festucae* and *E. typhina*, possibly because of their abundance in cultivated fields. If the interactions observed in Oregon represent a range expansion by endemic *B. lobata*, additional questions arise on the impact of the introduced fungi on the native *Botanophila–Epichloë* interactions. Further research is required for insights on the origin and host relationships of *B. lobata* in Oregon, and the impacts of exotic fungi on fly–fungus interactions on grasses in natural habitats.

Acknowledgements

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